Mitochondrial DNA Accumulation During Embryogenesis

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In The Name Of Allah

I wish to dedicate this work

To my great mother

To my beloved husband

To my dear country Saudi Arabia

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Abstract

Mitochondria play an important role in cellular energetic metabolism during all stages of life, including oocyte maturation, fertilization, and embryonic development. Mitochondrial DNA (mtDNA) replication occurs in growing oocytes, but stops once the oocyte reaches its full size (E. Mahrous *et al*., 2012). The timing and regulation of mtDNA accumulation in the embryo, however, remain poorly understood. Thus, the objectives of this study are to identify the precise timing of mtDNA replication resumption during mouse embryonic development and to investigate the molecular mechanisms controlling mtDNA accumulation during embryogenesis. In this study, we performed qPCR-based assays to estimate the quantity of mtDNA in individual mouse embryos at different stages of pre-implantation embryo development *in vitro*. We determined that mtDNA content remained constant up to the blastocyst stage. We then examined the expression of nuclear-encoded genes required for mtDNA replication. We found that the mRNA expression levels of *Tfam, Polga, Polgb, mtSSB, Peo1,* and *Nrf1* were very low or undetectable during most of the pre-implantation stages, but increased at the later blastocyst stage, which correlates with the number of mtDNA molecules that remained unchanged up to the blastocyst stage, and suggests the absence of mtDNA replication during this period. However, we also discovered that mtDNA copy numbers were significantly increased in the blastocyst outgrowth stage, which may suggest that the accumulation of mtDNA during embryogenesis is closely correlated with embryo growth. To investigate the mechanisms of mtDNA accumulation and assess whether the increase in mtDNA copy number during blastocyst outgrowth is dependent on embryo growth, we inhibited the mTOR pathway, which is essential for growth and cell proliferation in early mouse embryos (Murakami, Ichisaka *et al*., 2004). To do so, we cultured blastocyst outgrowths in the presence or absence of the mTOR inhibitor rapamycin and

quantified the mtDNA copy numbers. We found no significant difference in the content of mtDNA between the two groups, suggesting that the accumulation of mtDNA is independent of embryo growth. Moreover, using immunostaining methods, we assayed for active mtDNA replication in the blastocyst outgrowth stage. We observed potential mtDNA in the cytoplasm of the blastocyst outgrowths, supporting that mtDNA replication may take place at this stage of embryogenesis.

Résumé

Les mitochondries jouent un rôle important dans le métabolisme de l'énergie cellulaire durant toutes les étapes de la vie, incluant la maturation des oocytes, la fertilisation, et le développement embryonnaire. La réplication du génome mitrochondrial (ADNmt) se fait dans les oocytes en développement, mais se termine une fois qu'ils atteignent leur taille maximale. Le mécanisme de synchronisation et la régulation de l'accumulation de l'ADNmt dans l'embryon restent cependant des phénomènes peu explorés. Les objectifs de cette thèse sont donc d'identifier les temps précis de la reprise de la réplication de l'ADNmt durant le développement embryonnaire murin et d'étudier le mécanisme moléculaire qui régit l'accumulation de l'ADNmt durant l'embryogenèse. Dans cette étude, nous avons performé des analyses par amplification en chaîne par polymérase pour estimer la quantité d'ADNmt dans des embryons de souris individuels à différents stades de développement embryonnaire pré-implantatoires *in vitro*. Nous avons déterminé que le contenu de l'ADNmt restait constant jusqu'au stade de blastocyste. Nous avons ensuite examiné l'expression des gènes nucléaires requis pour la réplication de l'ADNmt. Nous avons trouvé que l'expression de l'ARN messager (ARNm) de *Tfam*, *Polga*, *Polgb*, *mtSSB*, *Peo1*, et *Nrf1* est faible ou indétectable durant la plupart des stades pré-implantatoires, mais qu'elle augmente au stade plus avancé du blastocyte, et que cela correspond avec le nombre de molécules d'ADNmt qui reste constant durant cette période. Cependant, nous avons aussi découvert que le nombre de copies d'ADNmt était significativement plus élevé durant l'excroissance au stade de blastocyste, ce qui suggère que l'accumulation d'ADNmt durant l'embryogenèse correspond avec la croissance de l'embryon. Pour étudier le mécanisme d'accumulation d'ADNmt et déterminer si l'augmentation du nombre de copies d'ADNmt durant

l'excroissance du blastocyste dépend de la croissance de l'embryon, nous avons inhibé la voie cellulaire mTOR qui est essentielle à la croissance et à la prolifération cellulaire dans l'embryon murin. Pour cela, nous avons fait des cultures cellulaires d'excroissances de blastocystes en présence ou en l'absence de l'inhibiteur de mTOR rapamycine et avons quantifié le nombre de copies d'ADNmt. Nous n'avons pas trouvé de différence significative dans le nombre de copies d'ADNmt entre les deux groupes, suggérant que l'accumulation d'ADNmt est indépendante de la croissance de l'embryon. De plus, nous avons tenté de détecter la réplication de l'ADNmt durant le stade d'excroissance du blastocyste en utilisant des méthodes d'immunofluorescence. Nous avons observé ce qui serait potentiellement de l'ADNmt dans les excroissances de blastocyste, supportant l'idée que la réplication de l'ADNmt pourrait se produire à ce stade de l'embryogenèse.

List of Abbreviations

µg……..…….....……..microgram

Actb……..…….……....actin, beta

ART…….....assisted reproductive technology

ATP……...……. adenosine-5'-triphosphate

BSA……...…….……bovine serum albumin

cDNA……...……....complementary deoxyribonucleic acid

ddH_2O……..……...double distilled water

DAPI ……. 4′,6-diamidino-2-phenylindole

DNA……..……..… deoxyribonucleic acid

DNAse……..…….……...deoxyribonuclease

eCG……..…equine chorionic gonadotropin

EdU……...…5-ethynyl-2′-deoxyuridine

ETC……..…….…electron transfer chain

Fmole……...……...…femtomole

GC……...……granulosa cells

hCG……...…… human chorionic gonadotropin

HRP ……..horseradish peroxidase

IP……..……. intraperitoneal

IPTG……..…Isopropyl β-D-1-thiogalactopyranoside

ITS……..……..…. Insulin-Transferin-Selenium

mRNA……..……..…messenger ribonucleic acid

mtDNA……..……..…..mitochondrial deoxyribonucleic acid

mtSSB …….mitochondrial single-stranded DNA-binding

mTOR……..…...….mammalian target of rapamycin

ND1…….....NADH dehydrogenase subunit 1

nDNA……..……..…nuclear deoxyribonucleic acid

NRF1……..…. nuclear respiratory factor 1

PCR……...…..… polymerase chain reaction

Peo1……. progressive external ophthalmoplegia

Pg……..……..…….picogram

POLG……..... mitochondrial-specific DNA polymerase gamma

Q-PCR……...……..…..quantitative polymerase chain reaction

RNA……..……... ribonucleic acid

TFAM……..…… mitochondrial transcription factor A

TSA.....tyramid signal amplification

X-Gal…….....5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

ZP……..……..……zona pellucida

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Preface & Contribution of Authors:

This work was completed under the supervision of dr.Hugh Clarke in collaboration with Enas Mahrous and myself.

Plasmid with rabbit glubin cDNA was prepared by Enas Mahrous at our lab under supervision of Dr. Hugh Clarke in previse research. She tested the recovery of rabbit globin cDNA that was added to each single embryo prior to DNA extraction.

My role was doing the whole experiments starting with super ovulating mice, collecting embryos and sample preparation .Our study also I did all the steps of DNA extraction, RNA extraction ,immunostaining process , imaging, data analysis and interpretation.

CHAPTER 1

INTRODUCTION

1.1 Fertilization and early stages of embryo development:

Embryogenesis is the process of cell division and cellular differentiation during early prenatal development which leads to the development of the embryo. In mice, fertilization occurs in the ampullae at the upper end of each oviduct where the eggs are located, approximately 2 hours after ovulation. Sperm are usually present in the ampullae at the time of ovulation. In the mouse, the first sperm to arrive at the egg usually penetrates and fertilizes it. The zona pellucida increases in thickness and changes following the entry of the first sperm in order to reduce the chance of subsequent penetration (Braden *et al*., 1958). Pronuclei, which are formed 4 ¼ hours after penetration, come together and fuse at the center of the zygote to form the zygote nucleus. Within 2 to 3 hours after fertilization, the second polar body is formed and is everted into the perivitelline space (Braden *et al*.,1962) (Austin and Braden .,1956) **(**Edwards and Gates *et al*., 1959). At the first cleavage, the nuclear walls break down, the chromosomes split longitudinally, and one-half of each split chromosome is diverted to each daughter cell. In mice, the first two cleavages occur while the eggs are still in the oviduct, and the first cleavage results in two cells which are nearly equal in size. Subsequent divisions occur more rapidly, giving rise to the four-cell stage, the eight-cell stage, and so on. Embryos of 16 cells or more, which are called morulae, generally reach this stage about 60 hours after fertilization and pass from the oviduct into the uterus (Lewis and Wright *et al*.,1935).

1.2 Blastocyst formation and implantation in utero:

Around the 16-cell to 32-cell stage of the morula, outer cells mature into an epithelium with tight junctions between cells and start to pump fluid from the outside into the embryo. The fluid accumulates in the intercellular spaces, and forms a cavity that enlarges rapidly, forming the blastocoel. This cavity is bounded by a single layer of cells except on one side where most of the cells are grouped to form a structure called the inner cell mass (ICM), with the outer epithelial cells forming the trophectoderm. The embryo is now a blastocyst that comprises approximately 200-300 cells. The trophoblastic cells will contribute to form the placenta, while the ICM will differentiate to form the fetus (Eaton and Green *et al.*, 1963) (Cross, Werb and Fisher *et a.*, 1994). Throughout the time from ovulation to implantation, the oocyte is surrounded by the pellucid zone. However, around day 6 after fertilization , the blastocyst emerges from the zona pellucida and directly contact with the endometrial epithelium (Red-Horse,Zhou *et al* ., 2004). The trophoblast cells then begin to multiply, forming two layers. The first is the syncytiotrophoblast layer, which forms the outer cell layer of the trophoblast and secretes proteolytic enzymes to break down the endometrial extracellular matrix, allowing for implantation of the blastocyst in the uterine wall. Blastocyst implantation is influenced by uterine and embryonic factors. Therefore the nature of the interaction between those factors determines the its success.(Carson, Bagchi and Dey *et al*., 2000). Eventually, the syncytiotrophoblasts come into contact with maternal blood and form chorionic villi, thus initiating formation of the placenta. The second layer is the cytotrophoblast layer, which consists of an irregular layer of ovoid, mono-nucleated cells directly below the syncytiotrophoblast. In normal pregnancy, cytotrophoblast cell proliferate and migrate along the Lumina of maternal spiral arterioles in which they replace the maternal endovascular lining such which enhances farther invasion that is essential to develop thefeto maternal vascular communication(Red-Horse,Zhou *et al* ., 2004).

1.3 Blastocyst outgrowth in vitro :

Culturing mouse blastocysts *in vitro* provides a good approach for examining the embryonic interactions with the microenvironment under highly controlled conditions. Major events that occur *in utero* can be followed *in vitro*, including blastocyst expansion, hatching, and adhesion to extracellular matrices. Developing blastocysts continue to accumulate fluid, and pressure inside the embryo increases. At the same time, proteolytic enzymes are produced by trophoblastic cells. This increase in pressure and production of proteolytic enzymes weakens the zona pellucida, producing an opening through which the trophectoderm eventually passes through in order to hatch and prepare for implantation. Around this time, the trophoblast migrates outside the zona pellucida and becomes adhesion-competent; the earliest sign of trophoblast outgrowth is the appearance of spreading cells near the base of the embryo. During the next 24 to 72 hours, the field of migrating trophoblast cells surrounding the embryo increases. The clump of cells at the center of the outgrowth consists of remnants of the ICM and undifferentiated trophoblast cells (Stachecki ,Yelian *et al*., 1994) (Osullivan ,Krpinka *et al*., 2002). Enabling cultured blastocysts to attach *in vitro* is in fact quite challenging and, as such, various approaches have been employed to enhance the attachment and adhesive phase of blastocyst outgrowth, which may represent cellular events associated with attachment to the luminal epithelium *in utero.* Normally, blastocysts are cultured on bovine serum albumin (BSA)-coated surfaces, allowing them to hatch from the zona pellucida and approach the attachment-competent stage. Unattached blastocysts will displace relative to debris or marks in the plastic, whereas attached embryos remain firmly in place. The time between the onset of attachment and the beginning of outgrowth can be as12 to 24 hours. Trophoblast cells are rapidly outgrowing on plates coated with fibronectin, laminin, vitronectin, collagens, entactin, and other components of the

extra cellular matrix (ECM) (Armant ,Kaplan *et al.*,1986)(Spindle, Pedersen *et al* .,1973). Some studies have shown that chemical removal of zona pellucida from day 5 in cultured human embryos is an effective and safe method of significantly improving the implantation rate (Jelinkova ,Pavelkova *et al* ., 2003).

The TOR (target of rapamycin) pathway is one of the signaling methods that may regulate embryo growth. TOR is a serine-threonine kinase belonging to the phosphoinositide kinase-related kinase (PIKK) family and was identified in *Saccharomyces cerevisiae* (Brown, Albers et al. 1994). Mammalian TOR (mTOR) is also known as rapamycin-associated protein (FRAP) (Kim, Sarbassov et al. 2002). TOR has been shown to be involved in the regulation of cell growth through cell size control. In *S.cerevisiae,* TOR acts to control cell size and proliferation (Barbet, Schneider *et al*., 1996). In *Drosophila melanogaster,* inactivation of the TOR homologue results in embryonic lethality associated with smaller cell sizes (Montagne, Stewart *et al*,. 1999). In the mouse, disruption of the mTOR pathway by deletion of the C-terminal of mTOR, resulted in reduced cell size and proliferation arrest in embryonic stem cells (Murakam,Ichisaka *et al.*, 2004). The TOR pathway is efficiently inhibited by the naturally occurring antifungal macrolide rapamycin (Ma and Blenis *et al.*, 2009), and treatment of mammalian cells with rapamycin results in reduced cell size. Together these data suggest that the mTOR pathway plays a critical role in cell growth control and proliferation in early mouse embryos.

1.4 Mitochondrial DNA:

Mitochondrial DNA (mtDNA) is double-stranded circular DNA of approximately 16,600 base pairs (bp) in humans and 16,300 bp in mice. Most of the sequences are coding sequences but there is also a non-coding region called the D-Loop, which includes the main regulatory sequences for replication and transcription.

In mice and most of multi-cellular organisms, mtDNA codes for 37 genes, including 13 protein-coding genes, 22 tRNA genes and 2 rRNA genes. The protein-encoding genes generate the 13 proteins of electron transport chain (ETC), 7 of which are complex I subunits, one is a complex III subunit, three are complex IVsubunits, and two are complex V subunit (Pon, Vestweber *et al*., 1989). The mtDNA copy number is in the range of 10^3-10^4 copies per somatic cell and is packaged in a protein-DNA complex called the nucleoid (Legros, Malka *et al*., 2004). Mitochondrial activities include multiple signaling pathways and cellular functions. In addition to generating most of the cell's ATP (Ernster and Schatz *et al.*,1981), mitochondria show an important role in Ca^{2+} homeostasis (Duchen *et al*., 2000) and intracellular metabolites (Brookes, Yoon *et al*,. 2004).

Mitochondrial transcription factor A (mtTFA or TFAM) and mitochondrial-specific DNA polymerase gamma (POLG) are important for regulating mtDNA replication (Clayton *et al*.,1982; Clayton *et al*., 1998). Another main factor is the nuclear respiratory factor 1 (NRF1), which activates the expression of genes regulating mtDNA replication such as TFAM (Virbasius and Scarpulla *et al*.,1994). It has been reported that mouse embryos from which NRF1 has been deleted exhibited markedly reduced mtDNA content (Huo and Scarpulla *et al.,* 2001), suggesting a key role for NRF1 in mtDNA biogenesis.

TFAM is a nuclear-encoded protein of the high-mnobility group (HMG) family. TFAM acts as a mtDNA packaging factor (Fisher *et al.*, 1992) and as a transcription factor, regulating both replication and transcription (Chang and Clayton *et al*., 1984). TFAM is thought to be essential for mtDNA replication, as inhibition of TFAM results in a decrease of mtDNA copy number, while over-expression of TFAM leads to an increase in mtDNA copy number. Similarly, cells with low mtDNA content contain undetectable TFAM expression (Ekstrand,

Falkenberg *et al*. ,2004), suggesting that mtDNA steady-state levels are regulated by TFAM. Specifically, TFAM is thought to package mtDNA in a manner analogous to histones and nuclear DNA (Alam, Kanki *et al*,. 2003) (Kanki, Ohgaki *et al*., 2004). Moreover, up-regulation of TFAM expression results in an increase in the expression of POLG (Ekstrand *et al.*, 2004), suggesting an important role for TFAM in initiating mtDNA replication.
POLG is a heterotrimer enzyme composed of one catalytic subunit (POLGA) and two accessory subunits (POLGB). As POLG is the only DNA polymerase found in mitochondria, it is responsible for mtDNA replication (Chan and Copeland *et al*.,2009). POLGA is the catalytic subunit that contains 3′–5′ exonuclease activity. POLGB is the accessory subunit that forms a heterodimer at a ratio of 2:1 with POLGA, thus stabilizing the enzyme to increase efficiency. In addition, a recent study has suggested that POLGB is the key factor that determines mtDNA copy number as it is required for D-loop synthesis (Di Re *et al*., 2009). *Polg* homozygous knockout mice manifest severe mtDNA depletion, respiratory chain deficiency, and embryonic lethality prior to day 8.5 (Hance, Ekstrand *et al*.,2005).

Two other factors play key roles in mtDNA replication, the Twinkle helicase (PEO1) gene and mitochondrial single-stranded DNA-binding protein (mtSSB). Twinkle displays 5′–3′ DNA helicase activity supporting its role in unwinding the mtDNA replication fork, and defects of this gene are associated with inherited mitochondrial disease such as progressive external ophthalmoplegia (PEO 1). In mice, increased expression of Twinkle in muscle and heart has been shown to increase mtDNA copy number up to 3-fold higher than controls. Additionally, reduced expression of Twinkle by RNA interference mediated a rapid drop in mtDNA copy number (Tyynismaa, Sembongi *et al.*, 2004). These data demonstrate that Twinkle helicase is essential for mtDNA maintenance, and that it may be a key factor of mtDNA replication in

mammals. Mitochondrial single-stranded DNA-binding protein mediates the unwinding of mtDNA through its physical interaction with Twinkle. In *Drosophila melanogaster*, mtSSB is required for mtDNA replication and development (Maier *et al*.,2000), thus further supporting that mtSSB plays a critcial role in mtDNA replication .

It has been demonstrated that mtDNA content of oocyte affects developmental competency , in other word low mtDNA content in oocytes is closely associated with fertilization failure, ovarian insufficiency, and poor oocyte quality (Reynier, May-Panloup *et al*.,2001; May-Panloup, Chretien *et al.*, 2005; Santos, El Shourbagy *et al*.,2006). Another study found that oocytes with low mtDNA content can be successfully fertilized and proceed normally through pre-implantation development, but die during the post-implantation stage, suggesting that oocyte and embryonic mtDNA is necessary for embryonic development (T. Wai *et al*., 2010). There is a critical threshold of mtDNA content in the oocyte to provide a minimum number of mtDNA molecules for each daughter cell during cell cleavage before mtDNA replication restart (M. Chiaratti *et al*., 2010). Oocytes with as few as 4000 copies of mtDNA can be successfully fertilized and progress normally though pre-implantation development up to the blastocyst stage. However, for post-implantion development, the minimal critical threshold of mtDNA increases to around 40,000 to 50,000 copies.

1.5 Variation of mtDNA content during early embryonic development:

The mtDNA is maternally inherited in animals and is exclusively transmitted by oocytes (Giles *et al*., 1980). In many species, extensive mtDNA replication occurs during oogenesis (Smith and Alcivar *et al*., 1993; Jansen and de Boer *et al*.,1998) (el Meziane, Callen *et al*., 1989) (Piko and Taylor *et al*.,1987; Ebert, Liem *et al*., 1988) (Vallejo, Lopez *et al.*,1996), and mtDNA steadily accumulates during oocyte growth. Once oocytes stop growing, the accumulation of mtDNA halts as well (E.Mahrous *et al*., 2012). MtDNA content during embryogenesis has been

examined in mouse embryos and shown to be constant from the oocyte stage to the blastocyst stage, using Southern blotting (Piko and Tylor *et al*., 1987) and quantitative PCR (Thundathil *et al*.,2005). These studies suggest that there is no mtDNA replication during the early stages of embryonic development following fertilization and before implantation. Rather, mtDNA replication resumes around the time of implantation, though the precise timing has not yet been defined (Thundathil, Filion *et al*., 2005). In bovine and porcine models, mtDNA content decreased between the 2-cell and 4/8-cell stage, then increased dramatically at the blastocyst stage (May-Panloup *et al*.,2005) (Spikings *et al*., 2007). During pre-implantaion development, mammalian embryos express either very low levels of mtDNA replication factors or no expression at all of these factors (Thundathil *et al*., 2005) (May-Panloup *et al*., 2005) (Spikings *et al*., 2007). In contrast, at the blastocyst stage the expression of mtDNA replication factors is upregulated, which leads to reactivation of mtDNA replication. At the same time, mitochondria start to differentiate into elongated organelles containing swollen cristae that are mostly located in trophoectodermal cells that will mediate the process of implantation.

1.6 Detection of mtDNA replication through immunostatining:

One approach to study if there is active mtDNA replication during embryogenesis is trying to detect the newly synthesized mtDNA in the cytoplasm during this period of development. Detection of DNA synthesis in proliferating cells relies on the incorporation of labeled DNA precursors into cellular DNA. The labeled DNA precursors, usually pyrimidine deoxynucleosides, are added to cells during replication and their incorporation into genomic DNA is visualized after incubation and sample staining. Common deoxynucleosides used for this purpose include [^{3}H]thymidine and 5-bromo-2′-deoxyuridine (BrdU). [^{3}H]Thymidine incorporation into DNA is usually detected by autoradiography, whereas detection of BrdU

is carried out using specific anti-BrdU antibodies. [^{3}H]thymidine was slow and the microscopic imaging of [^{3}H]thymidine-labeled DNA had a poor resolution. In contrast, BrdU immunostaining was both faster and gave better microscopic imaging of the labeled DNA. However, the main disadvantage of BrdU incorporation is that it requires DNA denaturation to expose the BrdU so that it may be detected with an anti-BrdU antibody. This denaturation step can disrupt dsDNA integrity, which can affect cell morphology and antigen recognition sites (Salic, Mitchison et al .2008)(Stephen ,Lentz et al 2010). Recently, the use of 5-ethynyl-2′-deoxyuridine (EdU) has been described to detect DNA replication. Detection of the EdU label is highly sensitive and can be accomplished in minutes. EdU is a nucleoside analog of thymidine and is incorporated into DNA during active DNA synthesis. Detection is based on a click reaction, a copper-catalyzed covalent reaction between an azide and an alkyne. EdU contains the alkyne and the Alexa Fluor dye contains the azide to form a very stable reaction product. One advantage of this method is that incorporation can be detected in dsDNA; because the fluorescent label is a small permeable molecule, it can easily access the non-denatured ds-DNA. Standard aldehyde-based fixation and detergent permeabilization are sufficient for the Click-iT detection reagent to gain access to the DNA. This is in contrast to BrdU assays that require DNA denaturation to expose the BrdU so that it may be detected with an anti-BrdU antibody. The Click-iT EdU assay uses biologically unique moieties, producing low backgrounds and high detection sensitivities. EdU is incorporated into active nuclear and mtDNA synthesis. Directly visualizing mtDNA synthesis can be challenging due to the weak signal obtained from the ~17 kb mitochondrial genome. In order to visualize nascent mtDNA, the Click-iT EdU assay requires signal amplification (Van Heusden, de Jong *et al.,* 1997). In our study, we coupled EdU incorporation into mtDNA together with tyramide signal amplification (TSA).

TSA is an enzyme-mediated detection method that utilizes the catalytic activity of horseradish peroxidase (HRP) to generate high-density labeling of a target antigen. In this technique, the target antigen is the fluorescent azide (Oregon Green® 488). It is used to identify the newly synthesized DNA through EdU incorporation. Using an anti-dye antibody (HRP-conjugated) followed by a secondary antibody–HRP conjugate in combination with a TSA analog with fluorescence emission comparable to the original fluorescent azide.

1.6 Objectives

The mechanisms that regulate mtDNA copy number in animal cells have not been fully elucidated. The first objective of this study was to determine the pattern of mtDNA accumulation, in particular the precise timing of mtDNA replication resumption during early embryonic development. To do so, we developed a PCR-based assay that allowed for the mtDNA content of individual embryos to be quantified. The second objective of this study was to investigate the molecular control of mtDNA replication in mouse pre-implantation embryos. For this, we assayed expression patterns of mtDNA replication factors throughout pre-implantation development *in vitro*.

CHAPTER 2
MATERIALS AND METHODS

2.1 Animal source:

In compliance with the regulations and policies of the Canadian Council on Animal Care, we performed all our experiments using CD1 female mice (Charles River Canada, St-Constant, Quebec, Canada) and approval form was obtained from the Animal Care Committee of the Royal Victoria Hospital (Protocol 5748).

2.2 Isolation of immature oocytes:

We removed the ovaries from CD1 female mice of 12-15 days old and transferred to HEPES buffered (pH 7.2) minimum essential medium (MEM-HEPES; Sigma) supplemented with sodium pyruvate (28 µg/ml; Sigma), pencillin G (63 µg/ml; Sigma), streptomycin (50 µg/ml; Sigma), and BSA (1 mg/ml; Sigma) at 37^{o}C. Then we placed the ovaries in MEM-HEPES medium supplemented with collagenase type I (1mg/ml; Worthington), trypsin (0.05%; Sigma) and DNAse (0.02 mg/ml; Sigma). After that ,ovaries were punctured using a 30-gauge needle then left for 30 min at 37^{o}C. We collected the oocytes surrounded by cumulus cells and in order to remove the cumulus cells repeated pipetting through a narrow-bore pipette was performed. Oocytes then were transferred into a new dish containing MEM-HEPES. Finally, single oocytes were placed individually into a 1.7ml microcentrifuge tube and store at -20^{o}C until day of analysis.

2.3 Isolation of 1-cell stage embryos:

CD1 female mice (4–6 weeks) received superovulation by intraperitoneal (Castrillon, Miao *et al.*) injection of 7.5 IU of equine chorionic gondotropin (eCG; Sigma) followed by 5 IU

of human chorionic gonadotropin (hCG; Sigma) 48 hrs later, and mated to CD1 males mice. On 16 hrs post-hCG injection, female mice CD1 were scarified and dissected. The 1-cell embryo masses were recovered by dissecting the oviducts and transferring them to collection medium (MEM-HEPES). In order to remove the cumulus cells we incubated the 1-cell embryo masses in phosphate-buffered saline(PBS) (pH 7.4) supplemented with 0.01% hyaluronidase (Sigma) for 3-5 min. Only those showing two clearly visible pronuclei were washed 3 times in fresh medium (MEM-HEPES) and used for the experiment. Single 1-cell embryos were individually transferred into a 1.7ml microcentrifuge tube, and the tube was kept at -20^{o}C until use.

2.4 Culture of pre-implantation embryos in vitro:

After recovering 1-cell stage embryos as described in the above section, the embryos were transferred in 50-µl drops of medium (KSOM from EmbryoMax, Cat#MR-020P-5F) under 1 ml mineral oil (Sigma embryo-tested light mineral oil). Twenty embryos were placed in each 50-µl drop of KSOM in 60mm tissue culture dishes. The complexes were cultured for 5 days at 37°C in a humidified atmosphere of 5% CO_2. In each experiment, embryos were graded at 48 hrs post-hCG at 40× magnification on a warmed microscope stage at ~37°C (Wild dissecting microscope), for the 1-, 2-, 4-, and 8-cell embryos, morula, and blastocyst developmental stages. In all experiments, embryos were observed and graded for the stage of development including compaction, blastocoel formation, and hatching. To collect 2-cell, morula, and late blastocyst stage embryos, these times corresponded to approximately 72, 96, and 120 hrs post-hCG in culture, respectively. Embryos from each stage were collected at these specific times and single embryos from each stage were transferred individually into a 1.7ml microcentrifuge tube, which were kept at -20^{o}C until use.

2.5 Zona pellucida removal protocol:

As mentioned above, blastocysts were collected from 1-cell stage embryos, which were cultured in 50-µl drops of medium (KSOM-EmbryoMax, Cat#MR-020P-5F) for 120 hrs post-hCG injection, . The manipulation was performed in 60 mm tissue culture dishes heated to 37°C under a dissection microscope at 40× magnification. Using a mouth-controlled pipette, we transferred blastocysts to a 30-µL drop of pre-warmed acidic Tyrode's solution (SIGMA, Taufkirchen, Germany) under mineral oil for approximately 10 seconds followed by careful washing in at least tree microdrops of KSOM.

2.6 Blastocyst outgrowths protocol:

For this, 100 µL of 0.1 % gelatin solution (Sigma-Aldrish,Cat#1393) was placed in each well of a 96-well suspension culture plate (Greiner Bio-one CELLSTAR, Cat#655185) for 5 min, then removed, and the plates were dried for 15 min. Blastocysts were then transferred using a mouth-controlled pipette from washing medium (KSOM) to culture medium (MEM-NAHCO3 supplemented with 20% foetal bovine serum, FBS) (Sigma-Aldrich,Cat#1207c). Single blastocysts were cultured in 100 µL of culture medium per well. The blastocysts were cultured for 3 days at 37°C in a humidified atmosphere of 5% CO_2. During the next 24-72 hrs, morphological changes were observed at 40× magnification on a warmed microscope stage at ~37°C (Wild dissecting microscope). The blastocysts became adherent to the surface of the culture plate and collapsed, while the trophoblasts were spread out surrounding the embryo, and the clump of cells at the center of the outgrowth consisted of remnants of the ICM and undifferentiated trophoblast cells.

Gene	Primers	Product size (bp)	Annealing temperature. (^{o}C)
Actb	5'-GCTGTGCTATGTTGCTCTAG-3' 5'-ATCGTACTCCTGCTTGCTGA-3'	445	55
mt-ND1	5'-CGATTAAAGTCCTACGTGATCTGA-3' 5'-CTGGGAGAAATCGTAAATAGATAGAAA-3'	80	60
mtSSB	5'-GTGGCGATCAGGGGATAGTG-3' 5'-ACGAATCATCCGTTCTATGCCT-3'	270	66
Nrf1	5'-TCTCACCCTCCAAACCCAAC-3' 5'-CCCGACCTGTGGAATACTTG-3'	254	57
Peo-1	5'-GGTTCAGACGGGATGCAGAA-3' 5'-ACACCTACACTGCCCTTTCG-3'	214	67
Polga	5'-CCTAAGCTCATGGCACTGAC-3' 5'-TGCTGCTTCCCCTGTTCAAG-3'	202	57
Polgb	5'-ACAGCAATCAGACACCC AG-3' 5'-TTCTATTGGCTCCTTTCCCC-3'	245	56
Rabbit globin	5'-GTGGGACAGGAGCTTGAAAT -3' 5'-GCAGCCACGGTGGCGAGTAT-3'	257	60
Tfam	5'-CATTTATGTATCTGAAAGCTTCC-3' 5'-CTCTTCCCAAGACTTCATTTC-3'	171	56

TABLE 1: Primer sequences used for quantification of mitochondrial DNA and of mRNAs encoding nuclear factors required for its replication.

2.7 Generation of standard curves for real-time PCR:

Corbett Rotor-Gene 6000 Application Software was used to generate a standard curve .We prepared serial dilution series (5 points, 10-fold dilutions) using the plasmids containing rabbit globin cDNA and mt-ND1 (see appendix A). Each dilution was run in triplicate and in each reaction a template negative sample was run as a control for non-specific amplification. To confirm that we generate only one real time PCR, we studied a melting curve. (See appendix B). In order to confirm the consistency of the results, three independent trials of the serial dilutions

were done. To normalize the variability between runs, one standard point of mt-ND1 and rabbit globin cDNA was included in the same reaction with the samples.

2.8 DNA Extraction from oocytes and embryos:

Before DNA extraction, we added one picogram (pg) of the plasmid (4.1 kb) containing the rabbit globin cDNA and 20 µg of glycogen to each 1.7-ml microcentrifuge tube containing a single oocyte or embryo. DNA extraction was processed using QIAamp DNA mini kits (Qiagen).We eluted samples twice with 50 µl sterile water. In order to avoid DNA degradation, samples were used for quantitative PCR on the day of extraction.

2.9 Absolute quantification of mt DNA copy number:

Real-time PCR was done on a Rotorgene 6000 cycler (Corbett, Montreal Biotech, Canada) using a 72-well rotor. The master mix in the reaction was composed of 10 µl FastStart Master SYBR Green Mix (Qiagen), 7 µl sterile ddH_2O, 1 µl of primers at a concentration of 10 µM and 2 µl of DNA template equivalents of 0.02 oocyte or embryo. The reaction was performed as follows: 95^{o}C for 5 min; denaturation in 40 cycles at 95^{o}C for 30 s, annealing at 60^{o}C for 30 s and elongation at 72^{o}C for 30 s. The final step of elongation took 7 min. At the end of each extension step (72^{o}C), SYBR Green fluorescence was read.

2.10 Primer design:

We ordered and purchased all primers from Sigma. Dr. Lawrence Smith (Université de Montréal, Faculté de médecine vétérinaire, CRRA, Canada) designed the primer sequences of mtND1 gene (Genbank NC 0050891). Primer sequences of *Polga*, *Polgb*, *Tfam*, and *Nrf1* were obtained from (Thundathil, Filion *et al*., 2005). Primer sequences of *mtSSB* and *Peo1* were obtained from Genbank . Primer sequences to detect rabbit globin cDNA were taken from (Adjaye, Herwig *et al*., 2007). *Actb* primer sequences were obtained from (Arnold, Francon *et al*.., 2008) (Table1).

2.11 Detection of mitochondrial DNA replication in blastocyst outgrowths:

Blastocysts were obtained and cultured singly, i.e. one blastocyst per well, in 96-well culture plates in 100 μl culture medium (MEM-NACO3) supplemented with 20% FBS, at 37°C in a humidified atmosphere of 5% CO_2 for 72 hrs (blastocyst outgrowth). On day 3 of blastocyst outgrowth, the culture medium was replaced with 100 μl of EdU working solution at a final concentration 10 μM, and the blastocyst outgrowths were incubated at 37°C in a humidified atmosphere of 5% CO_2 overnight. The next morning, blastocysts were fixed for 15 min at room temperature with 2% paraformaldehyde (PFA) in PBS, rinsed twice with 3% BSA in PBS, and permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature. EdU was labeled using the Click-iT EdU Microplate Assay Kit (Invitrogen, Cat#10214), following the manufacturer's instructions. The EdU reaction cocktail was prepared just prior to use by combining the Click-iT reaction buffer, Click-iT EdU buffer additive, $CuSO_4$, and Oregon Green® 488 azide. The permeabilization solution was removed and 100 μl of EdU reaction cocktail was added to each well and incubated for 30 min at room temperature, protected from light. The cocktail was removed, and blastocyst outgrowths were washed twice in 3% BSA in PBS for 5 min each time.

The Oregon Green® 488 signal was amplified with a Tyramide Signal Amplification (TSA) kit (Invitrogen, Cat#T20989), and blastocyst outgrowths were incubated with an anti-Oregon Green antibody (diluted 1:200 in blocking buffer from the TSA Kit) overnight at 4°C with gentle rotation. The next morning, blastocyst outgrowths were rinsed three times in 3% BSA in PBS for 5 min each time, followed by incubation with a HRP-conjugated rabbit antibody against Oregon Green (from the EdU Microplate Assay Kit), diluted 1:300 in blocking buffer from the TSA Kit (Invitrogen, Cat#T20989), for 1h at room temperature. Blastocyst outgrowths were then washed

three times in 3% BSA in PBS. During this incubation period, the cocktail amplification buffer was prepared at a final concentration of 0.0015% H_2O_2 in amplification buffer from the TSA Kit. Also, 1 μL of Fluoresent Tyramide reagent from the TSA Kit, 1 μL 4',6-diamidino-2-phenylindole (DAPI) and 1 μL DRAQ5 were added to the cocktail amplification buffer. DAPI and DRAQ5 were used to stain the nuclear DNA. Blastocyst outgrowths were then incubated with Alexa Fluor 488–labeled tyramide at 1:100 in amplification buffer, plus 0.0015% H_2O_2 for 30 min at room temperature, and rinsed three times in 3% BSA in PBS. Confocal images were analyzed and collected using a Carl Zeiss LSM510 Confocal Imaging System.

2.12 Inhibition of blastocyst outgrowth:

In order to test whether mtDNA accumulation is dependent on embryo growth, we inhibited blastocyst growth by using the mTOR pathway inhibitor rapamycin (Sigma) at a working concentration of 100 nM. Rapamycin was added to the culture medium for 24 hrs. An equal volume of DMSO was added to non-treated samples as a control. DNA extraction for a total of 10 blastocyst outgrowths from each group was performed to quantify the mtDNA copy from each blastocyst outgrowth. Single blastocyst outgrowths were cultured in individual wells of a 96-well plate, and 1 pg of the plasmid (4.1 kb) containing the rabbit globin cDNA and 20 μg of glycogen was added to each well. This allowed us to determine the efficiency of DNA purification, based on recovery of rabbit globin cDNA, and mtDNA copy number for each sample. DNA extraction was performed using QIAamp DNA mini kits (Qiagen). Samples were eluted twice with 50 μl of sterile water and assessed by quantitative PCR on the day of extraction or no later than the following day to avoid degradation.

2.13 RNA extraction & complementary cDNA synthesis:

A total of 60 samples from each of 15 day-old oocytes, 2-cell stage embryo, morula and blastocyst were isolated and transferred to different 1.7-ml microcentrifuge tubes. Before RNA extraction 9 pg of rabbit mRNA (Sigma) was added as an external control to each tube.RNA extraction was performed using (PicoPure, Arcturus, Biosciences Mountain View, CA), and reverse-transcribed using SuperScript II Reverse Transcription kit (Invitrogen) producing a final volume of 50 μl of RNA. RT-PCR was processed using a Rotorgene 6000 cycler. RT-PCR cycle conditions were conducted as above described with modification in the annealing temperature according to the gene was amplified (*Tfam* and *Polgb* at 56^{o}C; *Nrf1*; *Polga* at 57^{o}C;*mtSSB* at 66 ^{o}C and *Peo1* at 67 ^{o}C). The double stranded cDNA was identified with SYBR Green, which was in the content of in the FastStart SYBR Green Master I Mix (Qiagen). For PCR we used a complementary DNA equivalents of 0.5 embryos. In order to confirm the absence of genomic DNA contamination a control of embryo after the DNAse I treatment using RNase-Free DNase Set (Qiagen), without the RT step was tested with other samples in the PCR. In each reaction a template negative sample was run as a control for non-specific amplification. Primer sequences were obtained from (Thundathil, Filion *et al*., 2005)(Genbank) (Table 1). The formula $2^{-(CT \text{ of sample} - CT \text{ of rabbit globin})}$ was used to calculate the relative mRNA expression of interesting genes, where C_T (cycle count) is the threshold cycle value.

2.14 Statistical analysis:

The Student's *t*-test and one-way ANOVA followed by Tukey's honest significance difference were used to analyze the data and aprobability value of < 0.05 was considered statistically significant. All experiments were repeated three times. All values presented are mean ± SEM.

CHAPTER 3

RESULTS

3.1 Using plasmid DNA to normalize for mtDNA extraction in single embryos:

One of the challenges of quantifying mtDNA copy number in single embryos using PCR is choosing an appropriate control to normalize differences in DNA extraction efficiency (E.Mahrous *et al*., 2010). In our study, we used a plasmid containing rabbit globin cDNA as an external control. This plasmid s is a circular DNA molecule, and so is likely to be recovered with similar efficiency to the mtDNA . Plasmid DNA was added to the tube containing a single embryo prior to DNA extraction. Plasmid DNA was recovered at an efficiency of ~ 80% **(E.**Mahrous *et al* , 2010).

3.2 MtDNA copy number remains constant during embryogenesis up to the blastocyst stage:

To establish the pattern of mtDNA copy number during embryogenesis, we collected mice embryos at different developmental stages, from 1-cell, 2-cell, morula, and blastocyst stages. Plasmid DNA was added, then DNA was extracted, and each sample was used to qPCR. As shown in **Figure 1**, we found that the content of mtDNA remained stable during embryo development.

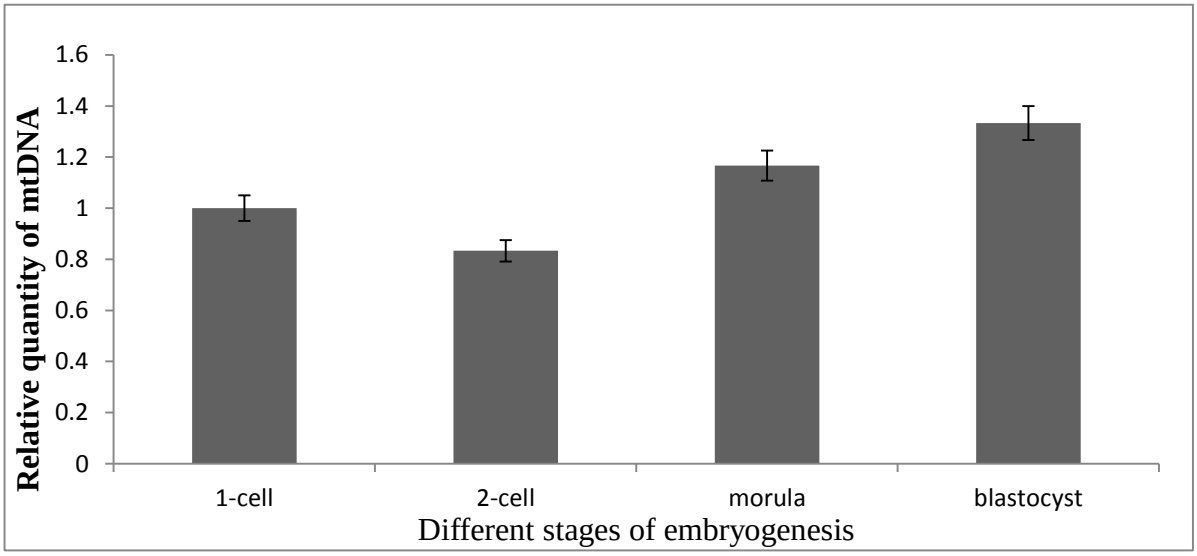

Figure 1: Comparison of mtDNA content in mice embryos at different stages during embryogenesis. The number of embryos in each group is 10. (ANOVA, Tukey, $p < 0.005$).

3.3 Nuclear–encoded mitochondrial replication factors (Tfam, Nrf1, Polga, Polgb, mtSSB, Peo1) may be involved in the regulation of mtDNA replication during embryogenesis:

MtDNA replication requires several nuclear-encoded proteins, including the mitochondrial transcription factor TFAM (Larsson, Wang *et al*.,1998), the nuclear respiratory factor 1 (NRF1) (Virbasius and Scarpulla *et al.*,1994), and DNA polymerase γ (POLG) (Graziewicz, Longley *et al*., 2006). Twinkle helicase (PEO1) is essential for mtDNA maintenance and may be a key factor in mammalian mtDNA replication (Tyynismaa1, Sembongi *et al*., 2004**).** Mitochondrial single-standard binding protein (mtSSB) is required for mtDNA replication and development (Maier *et al*.,2000). Embryos at different stages of growth were obtained from mice and the amount of the encoding mRNAs was quantified using qPCR.

Actin B (Actb) expression was measured as an internal control, but our results indicated that its expression pattern varied significantly during the period of pre-implantation development (**Figure 2**). Other housekeeping genes (GAPDH and HistoneH2A) were tested in a previous study and gave similar results (Thundathil *et al*., 2005). Since none of the above housekeeping genes showed stable levels of mRNA throughout pre-implantation development, a known amount of an external mRNA of rabbit globin was added to the embryo prior to carrying out the reverse transcription (RT) reactions, this is one of good approach that had been used as an external control .

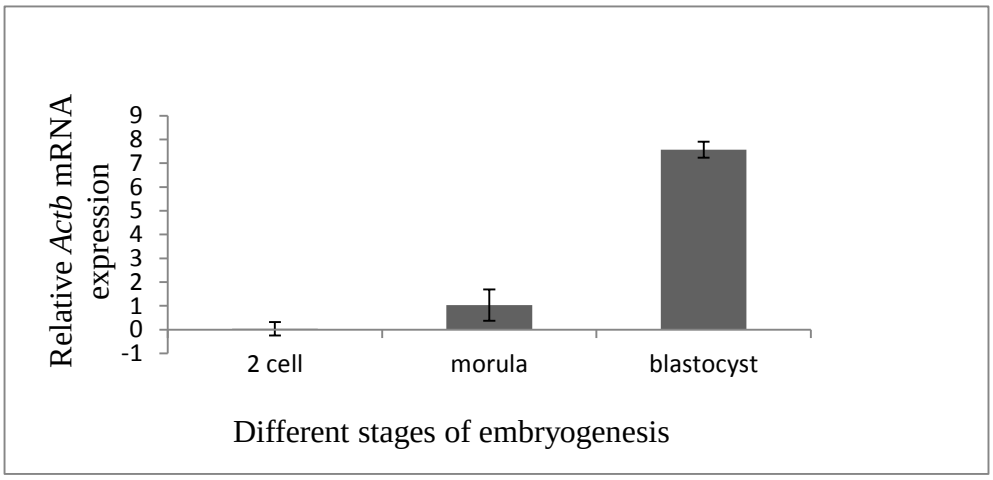

Figure 2: Expression patterns of the housekeeping gene Actb in pre-implantation embryos. The x-axis indicates the different stages of embryo development and the y-axis indicates the relative amount of *Actb* mRNA.The experiment was performed in triplicate using three pools of embryos. ($p < 0.05$).

The *Polga, Polgb, mtSSB, Tfam,* and *Peo1* mRNA expression levels were very low at the 2-cell embryo stage, but increased at the morula stage, and then increased sharply at the blastocyst stage. *Nrf1* transcripts remained relatively constant after fertilization and in the morula stage, but increasedat the blastocyst stage (Figure3).

Together, these results indicate that the mtDNA replication machinery is activated during pre-implantation development even though there is no significant increase in mtDNA copy number until after implantation.

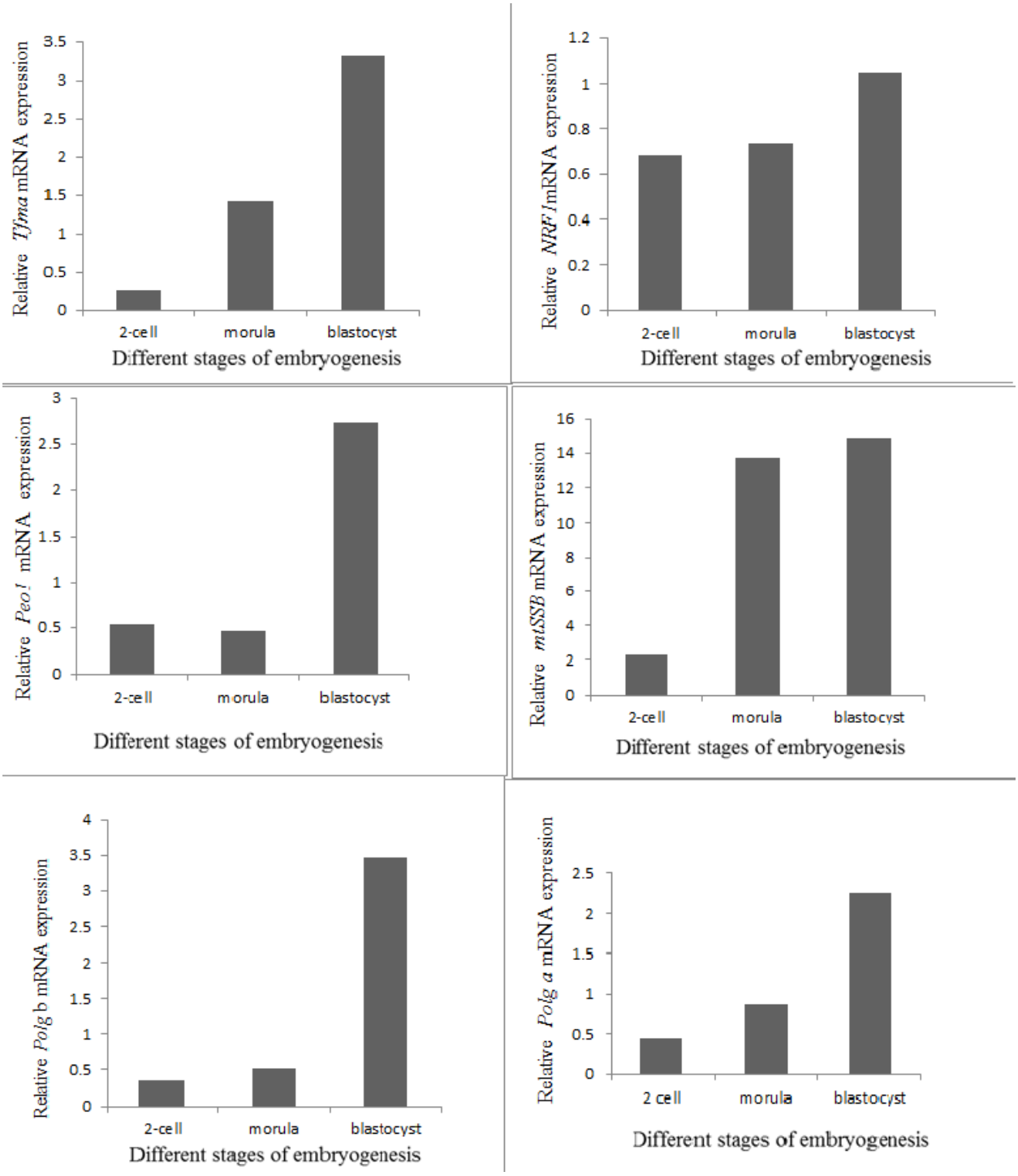

Figure3: Expression of factors required for mitochondrial DNA replication during different stages of embryo development *in vitro*.

3.4MtDNA copy number increased progressively at blastocyst outgrowth stage:

We collected blastocyst and blastocyst outgrowths. Plasmid DNA was added, DNA was extracted, and each sample was subjected to qPCR. As shown in **Figure 4**, we found the content of mtDNA increased significantly at the blastocyst outgrowth stage.

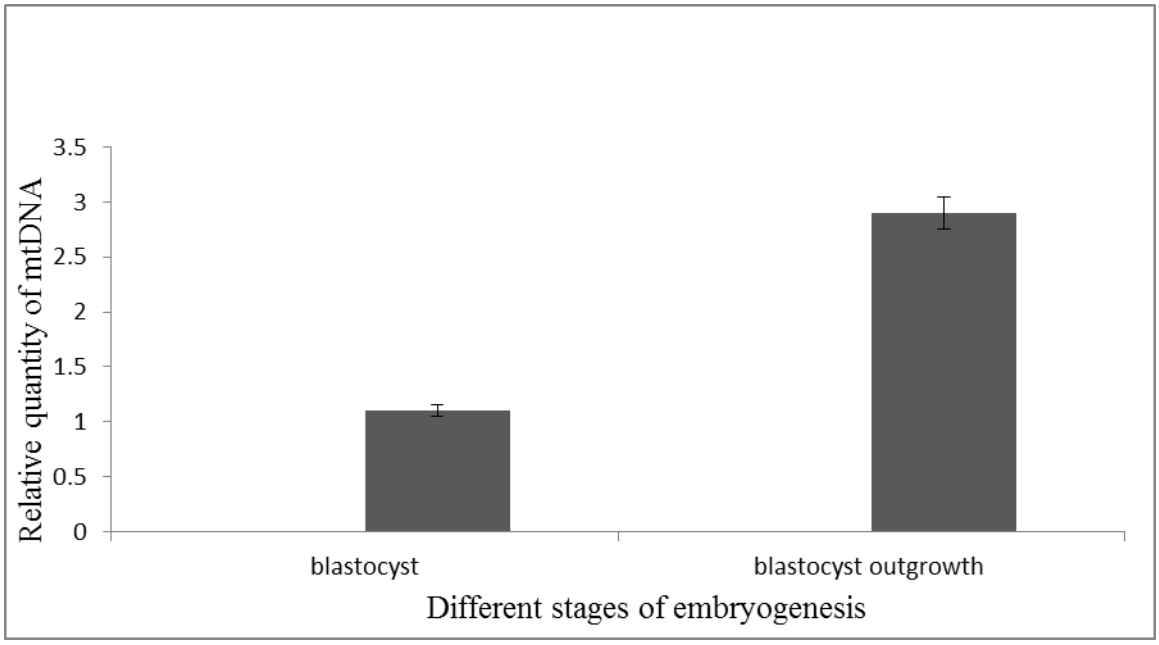

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Figure 4: Mitochondrial DNA content in blastocyst and blastocyst outgrowth .

Mitochondrial DNA copy numbers are significantly increased in blastocyst outgrowth. The number of embryos in each group is 10. (*t*-test, $p < 0.0001$).

3.5 Embryo growth and mTOR activity are not required for mtDNA accumulation:

To test whether the increase in mtDNA copy number was dependent on embryo growth, we inhibited the mTOR pathway, which is essential for growth and cell proliferation in early

mouse embryos (Murakami, Ichisaka et al. 2004). Blastocyst outgrowths were collected and cultured in the presence or absence of the mTOR inhibitor rapamycin for 24 hours. Plasmid DNA was added, DNA was extracted, and each sample was subjected to qPCR. As shown in **Figure 5**, we found that the content of mtDNA remained stable and that there was no significant difference in mtDNA content between these two groups. Surprisingly, rapamycin treatment did not impair mtDNA accumulation.

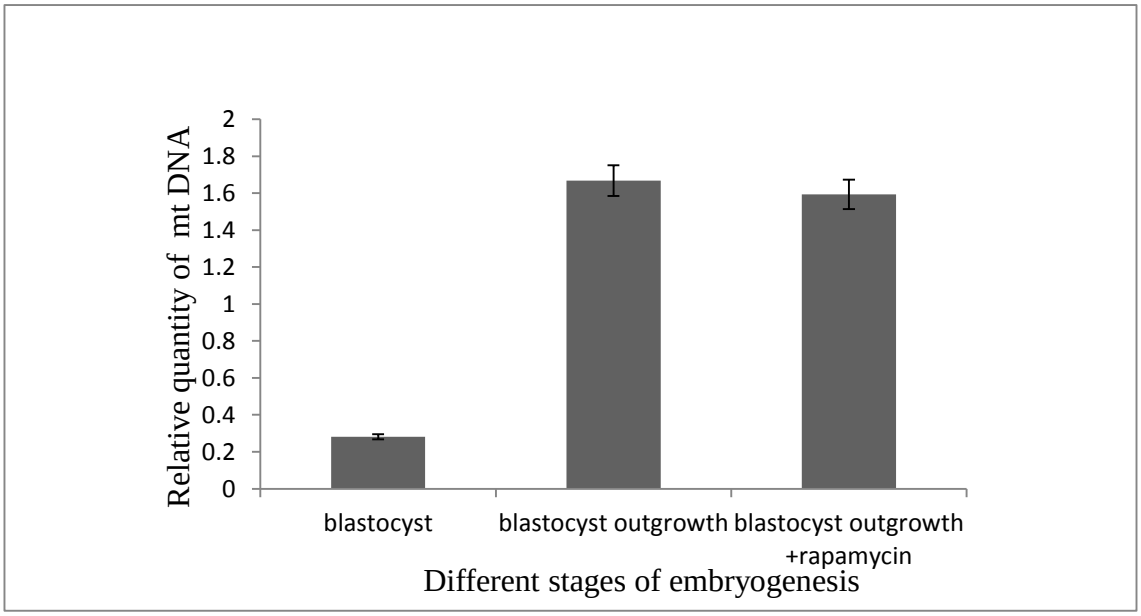

Figure 5: Effect of the absence of mTOR on blastocyst outgrowth and mtDNA accumulation *in vitro*.

Blastocysts were isolated and mtDNA content of one group of themere measured (day 0). The remaining blastocysts were cultured for 3 days to reach the blastocyst outgrowth stage. These were then cultured for 24 hours in the presence or absence of rapamycin (100 nM), and mtDNA copy numbers were measured. The number of embryos in each group is 10. (ANOVA, Tukey, $p < 0.005$).

To confirm the effectiveness of the rapamycin we verified that the phosphorylation of ribosomal protein S6 (p-s6RP) was inhibited in the treated oocytes (results not shown)**.** These results suggest that mTOR function may not be required for mtDNA accumulation.

3.6 Detecting newly synthesized mtDNA in the blastocyst outgrowth stage through immunostaining:

(I)5-ethynyl-2'-deoxyuridine (EdU) incorporation into nuclear and mitochondrial DNA of blastocysts without signal amplification (Figure 6). Blastocyst outgrowths were incubated with EdU for 24 hrs. EdU signal (green) is compared with DAPI (blue) without signal amplification.

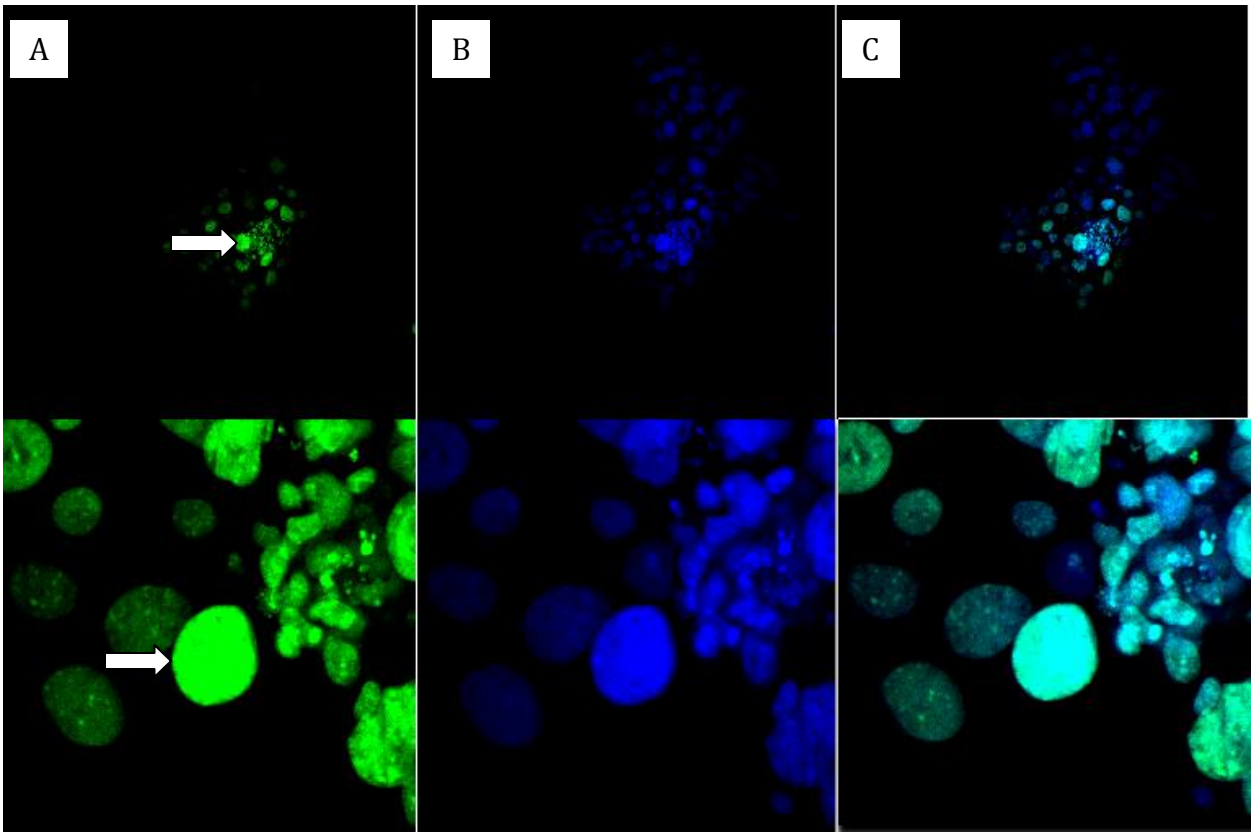

Figure 6: EdU incorporation into nuclear and mitochondrial DNA of blastocyst outgrowths without signal amplification (A) Standard immunofluorescence shows nuclear EdU (white arrowhead) signal but weak or no signal in mtDNA. (B) Nuclear DNA stained with DAPI. (C) Merge.

(II) 5-ethynyl-2´-deoxyuridine (EdU) incorporation into nuclear and mitochondrial DNA of blastocysts with Tyramide Signal Amplification TSA (Figure 7). Blastocyst outgrowths were incubated with EdU for 24 hrs. EdU signal (green) is compared with DAPI (blue) with TSA.

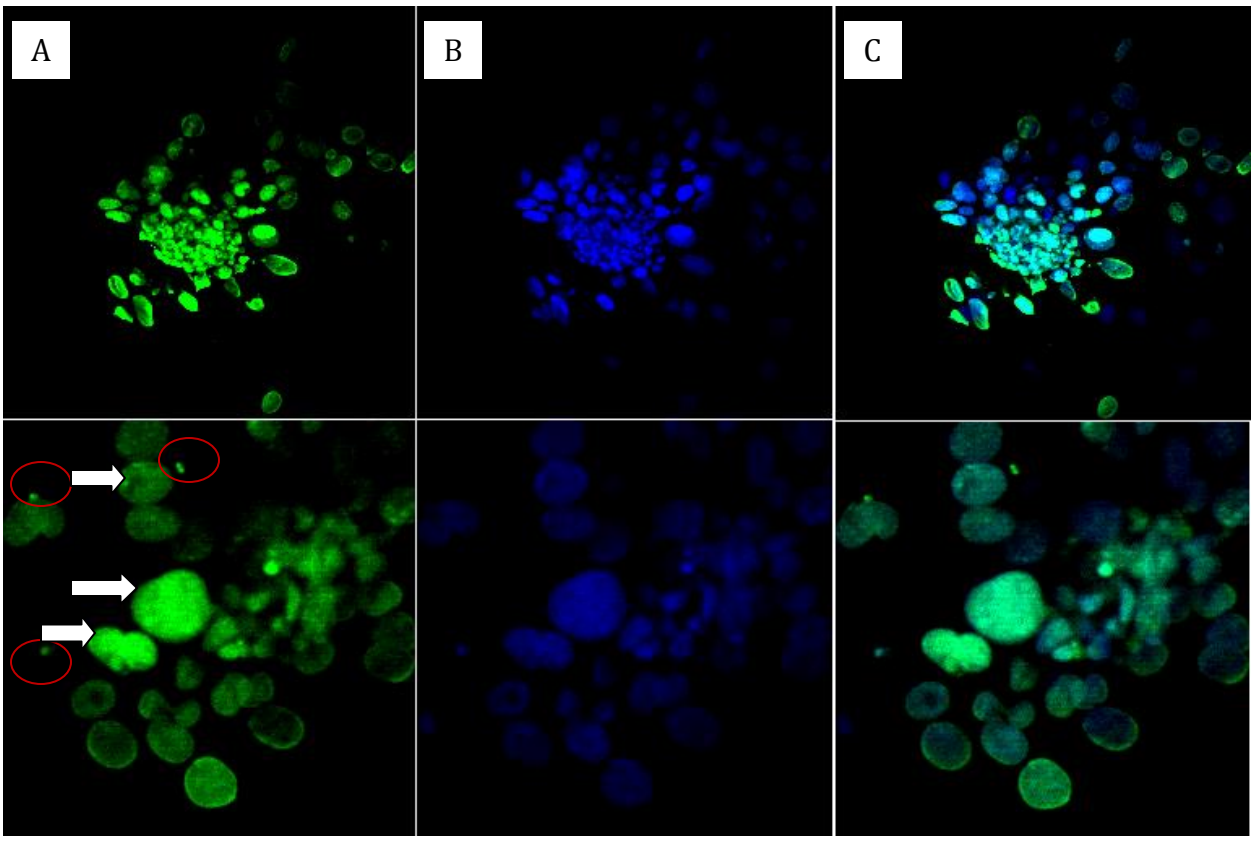

Figure 7: EdU incorporation into nuclear and mitochondtrial DNA of blastocystout growth with TSA.

(A) Immunofluorescence shows both nuclear DNA (white arrows) and mitochondrial DNA (red circles) incorporation. (B) Nuclear DNA stained with DAPI. (C) Merge.

CHAPTER 4
DISCUSSION

4.1 Using a recombinant plasmid as an exogenous reference for measuring mtDNA copy number:

Large variations in mtDNA copy number in individual oocytes have been reported (Piko and Matsumoto *st al*.,1976; Steuerwald, Barritt *et al*. ,2000; Thundathil, Filion *et al.*, 2005). The variation between these different studies could be due to several factors, including the efficiency of the mtDNA extraction. Choosing an adequate control is the most important step in the design of quantitative mtDNA experiments. In our study, we used a recombinant plasmid containing rabbit globin cDNA as an exogenous control. This method was developed in our lab and prepared by Enas Mahrous (E.Mhrous *et al*., 2010). Rabbit globin cDNA was chosen because highly specific and efficient qPCR primers had previously been identified (Adjaye, Herwig *et al*., 2007). This plasmid was recovered at ~ 80% efficiency with little variation, suggesting that the DNA was extracted with relatively equal efficiency from most embryos (E,Mahrous *et al*.,2010).

4.2 MtDNA accumulation during embryogenesis:

In our study, we found that mtDNA content does not significantly increase during early embryo development up to the blastocyst stage, which is consistent with previous findings from other studies that showed that mtDNA content remains constant during early development from the oocyte to the blastocyst stage (Piko and Taylor *et al*.,1987), (Thundathil *et al*., 2005). These results suggest the absence of mitochondrial replication before implantation. However, after

fertilization, embryo cleavage leads to mtDNA segregation among daughter cells, thus, the number of mtDNA molecules per cell decreases after each cell cycle, leading to post-implantation developmental arrest if the starting copy number is below the threshold (40,000-50,000). Developmental arrest is thought to be caused by dilution of mtDNA copies below a minimum number necessary to maintenance the cellular energy requirement (Chiaratti *et al*., 2010). If there is in fact no mtDNA replication during this period, then we would expect to see a decrease in the mtDNA content following embryo cleavage. However, the unchanged levels of mtDNA that we observe may reflect a balance between the segregation and the neosynthesis of mtDNA. That is, mtDNA replication may start at some point during pre-implantation development. In a study using bovine embryos, there was no change in the mtDNA content between metaphase II and 2-cell stage embryos, but a significant decrease in mtDNA content between 2-cell stage and 4/8-cell stage embryos. The mtDNA copy numbers then remained constant until dramatically increasing at the blastocyst stage (May-Panloup *et al*., 2005). Another study established small mtDNA replication events at pronuclear and 2-cell stage mouse embryos (McConnell and Petrie *et al*.,2004). At early embryonic development, spherical immature mitochondria with few cristae are located in the perinuclear regions of the cytoplasm (Von Blerkom *et al*.,2004). However, at the blastocyst stage, mitochondria start to differentiate into elongated organelles having swollen cristae (Sterm *et al*.,1971;Piko and Matsumoto *et al*.,1976;Wilding *et al*.,2001), and acquire a higher oxygen consumption and increased OXPHOS activity, leading to ATP production (Trimarch *et al* .,2000)(Wilding *et al*.,2001). MtDNA replication is mostly specific to the trophoectodermal cells, which are no longer pluripotent and typically express markers such as Cd2 (Niwa *et al*., 2005). These cells give rise to the placenta and facilitate the process of implantation if the embryo continues to develop. In order to

understand why mtDNA is mostly increased in trophoectodermal cells and not the ICM, a previous study investigated the relationship between pluripotency and mtDNA proliferation during early embryo development and embryonic stem cell differentiation (J. M. Facucho-Oliveira, St. John *et al.*, 2009). Early pre-implantation embryos and undifferentiated embryonic stem cells (ESCs) contain small and immature mitochondria located around the nucleus and have low oxygen consumption with high expression levels of pluripotency genes such as Oct4, Nanog and Sox (Chen and Daley *et al.*,2008). As such, the high expression levels of these pluripotency genes are associated with low expression levels of mtDNA replication factors. However, once embryonic cells and ESCs lose pluripotency, the expression level of mtDNA transcription and replication factors is increased and the quantity of mitochondria and mtDNA copies/cell also increases (J. M. Facucho-Oliveira, St. John *et al.*,2009).

4.3 MtDNA mtDNA accumulation is correlated, yet independent of embryo growth:

Our results have shown a progressive increase in the amount of mtDNA content at the blastocyst outgrowth stage day 4 *in vitro* relative to the mtDNA content at the blastocyst stage. Based on these observations, we proposed a relationship between blastocyst growth and mtDNA copy number accumulation. In order to test this hypothesis, we examined blastocyst outgrowth *in vitro* in the presence or absence of growth inhibitor. For this experiment, we used the mTOR inhibitor rapamycin. Our interest in the potential role of the mTOR pathway on mtDNA accumulation was based on its involvement in the regulation of cell growth (control of cell size) in many different species such as *S.cerevisiae* (Barbet, Schneider *et al*., 1996; Di Como and Arndt*et al.*, 1996) and *Drosophila melanogaster* (Oldham, Montagne *et al*., 2000; Zhang, Stallock et al. 2000; Loewith,

Jacinto *et al.* ,2002), as well as in the mouse (Shima, Pende *et al*., 1998). It has been reported that mTOR is essential for growth and proliferation in early mouse embryos and embryonic stem cells (Murakami, Ichisaka *et al*., 2004). In our study, we found no significant difference in the mtDNA content after rapamycin treatment. However, previous studies have shown that rapamycin treatment of blastocysts did not impair inner cell mass (ICM) proliferation whereas it inhibited trophoblast growth (Murakami, Ichisaka *et al.*, 2004). Despite inhibiting growth, mtDNA accumulation is not inhibited. In conclusion, these results suggest that mtDNA accumulation is not dependent on embryo growth.

4.4 MtDNA replication factors pattern during embryogenesis:

To study the molecular mechanisms that control mtDNA replication, we investigated the mRNA expression levels of specific nuclear-encoded genes that control mtDNA replication. Our results are consistent with other reports from previous studies that showed that during the early cleavage stages, mammalian embryos express either very low levels of mtDNA replication factors or there is no expression (May-Panloup *et al*.,2005), (Piko and Taylor *et al*.,1987) , (Thundathil *et al*.,2005). However, at the blastocyst stage, the expression of mtDNA replication factors is upregulated, which leads to reactivation of mtDNA replication.

A number of studies have defined the role of nuclear-encoded genes, such as *Polg* and *Tfam*, in mtDNA transcription and replication (Ekstrand, Falkenberg *et al*.,2004; Kang, Kim *et al*., 2007; Pejznochova, Tesarova *et al.*, 2010). A previous report using mice with either *Tfam* overexpression or *Tfam* knockout has demonstrated that mtDNA copy number is directly proportional to the quantity of TFAM protein in a variety of differentiated mouse tissues and E8.5-E9.5 mouse embryos (Ekstrand, Falkenberg *et al*., 2004). We considered that mtDNA

accumulation during embryogenesis might be limited by the quantity of these replication factors. To test this hypothesis, we analyzed the mRNA levels of *Nrf1*, *Polga*, *Polgb*, mtSSb, *Peo1*and *Tfam* during embryo development.

In general, pre-existing mRNAs, proteins, and organelles present in oocytes before fertilization are critical components for early embryonic development before the genome of the embryo begins to be expressed, thus the embryo itself mediates its own development. As other studies showed that mitochondrial transcriptional activity appears to occur at the same time as maternal-embryo transition (MET). In the present study, we have shown that mRNA levels of *Tfam, Peo1, mtSSB,* and the two subunits *Polga* and *Polgb* increased at the morula-blastocyst stage. In contrast, the mRNA levels of these genes decreased in the early developmental stages. The low levels of *Tfam, Peo1, mtSSB, Polga,* and *Polgb* mRNA expression at the 2-cell stage may have resulted from the degradation of stored maternal mRNA, as has been reported in other species (Robert *et al*., 2002), or may have been due to the absence or low level of embryo gene expression. While there are significantly increased amounts of transcripts of *Tfam, Peo1, mtSSB, Polga,* and *Polgb* in the morula and blastocyst stage embryos, a concurrent increase in mtDNA copy number was not detected. These results suggest that the stable mtDNA copy numbers in pre-implantation embryos is not due to the lack of expression of genes encoding mtDNA replication factors. Alternatively, it is possible that there is active mtDNA replication but also concomitant mtDNA degradation, thus stabilizing the total mtDNA amounts during this period. Moreover, post-transcriptional control mechanisms could also affect the stability of mtDNA copy number during pre-implantation embryo development. Further studies are required to determine whether the presence of *Tfam, Peo1, mtSSB, Polga,* or *Polgb* mRNA would stimulate mtDNA replication.

In comparison to the other genes tested, *Nrf1* transcripts were found to be at higher levels in early embryo development and maintained relatively constant up to the morula stage, after which *Nrf1* transcripts increased significantly at the blastocyst stage. In the mouse model, *Nrf1* transcripts also pre-exist in metaphase II oocytes and were found to remain at a steady state from the 8-cell stage to the blastocyst stage (Thundathil *et al*., 2005). The pre-existence of maternal *Nrf1* mRNA before MET activation could therefore be essential to the mtDNA activation pathway and play an essential role in triggering mitochondrial biogenesis. This is likely due to the fact that NRF1 has a broader role in the integration of multiple cellular functions (Huo and Scarpulla *et al*.,2001), including mtDNA maintenance (May-Panloup, Vignon et al. 2005) and transcription of nuclear-encoded genes (Patti, Butte *et al*., 2003; Scarpulla 2008).

4.5 Detecting mtDNA replication through immunostaining :

In other studies, mtDNA replication was assessed by BrdU staining, which takes place in pronuclear and 2-cell stage mouse embryos (McConnell and Petrie *et al*., 2004). In this study, we employed a new immunostaining approach to detect newly synthesized mtDNA molecules. We used a new technique that combines the Click-It EdU assay with Signal Amplification Technology designed for the sensitive and reliable measurement of nascent mtDNA synthesis. EdU was used to determine its effectiveness at labelling newly synthesized mtDNA. EdU incorporation into mtDNA is a significant improvement in this technique because standard BrdU epitope retrieval protocols are harsh and require acid or DNase treatments of the cells or tissue. While EdU incorporation into mtDNA was difficult to visualize with standard immunofluorescence, a substantial improvement in signal strength was obtained with the use of TSA. In our experiments, blastocyst outgrowths incubated with EdU for 24 hours demonstrated clear nuclear DNA but no mitochondrial DNA labeling (**Figure 8**). However, the tyramide-amplified EdU signal was visible in both nuclear DNA and mtDNA (**Figure 9**). With signal amplification, we observed a signal in the cytoplasm of blastocyst outgrowths, which we propose to be the newly synthesized mtDNA molecule. These results suggest that mtDNA replication may restart at this stage of embryogenesis.

In future, we will use the Mitotracker to determine the mitochondria and try to co-localize with the signal that we observed in this experiment.

Concluding statement:

In the present study, we provided a detailed characterization of mtDNA accumulation pattern during embryogenesis, showing that the mtDNA content remains constant up to the blastocyst stage. Moreover, we demonstrated that mtDNA accumulation during embryogenesis is independent of embryo growth. We further demonstrated that expression levels of nuclear-encoded mtDNA replication factors increased at the morula and blastocyst stage, though mtDNA replication did not occur until the blastocyst stage, suggesting that the inhibition of mtDNA replication is controlled at the post-transcriptional level during early embryogenesis. Finally, we used a new immunostaining approach to detect newly synthesized mitochondrial DNA at the blastocyst outgrowth stage.

Future study:

Our imaging showed a potential of replication at blastocyst outgrowth stage, in order to be more specific to identify mtDNA replication in the cytoplasm of the blastocyst outgrowths, I am planning to use mitotracker as a confirmation step.

From other side, the way to understand the molecular mechanism that control mtDNA replication during embryogenesis, I would like to study the pattern of mtDNA replication factors at blastocyst outgrowth stage.

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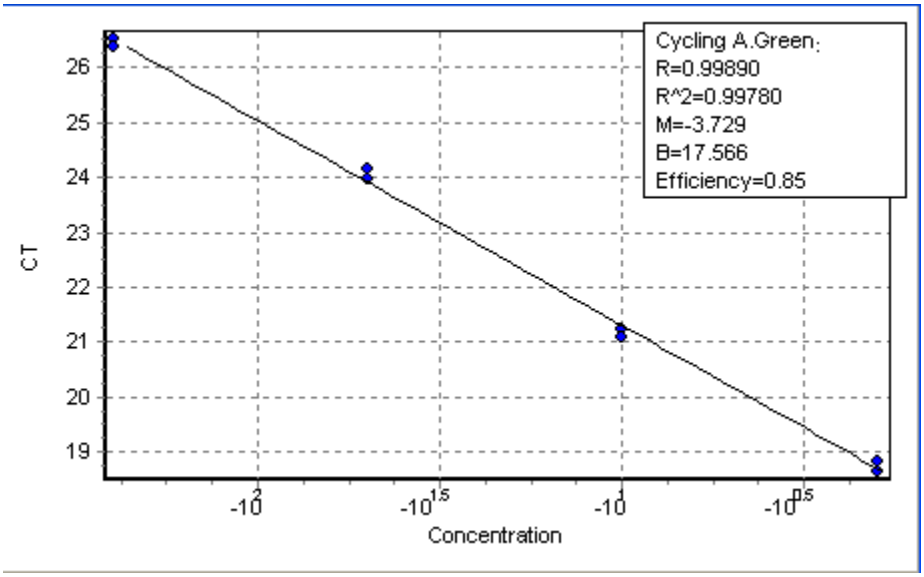
Appendix A

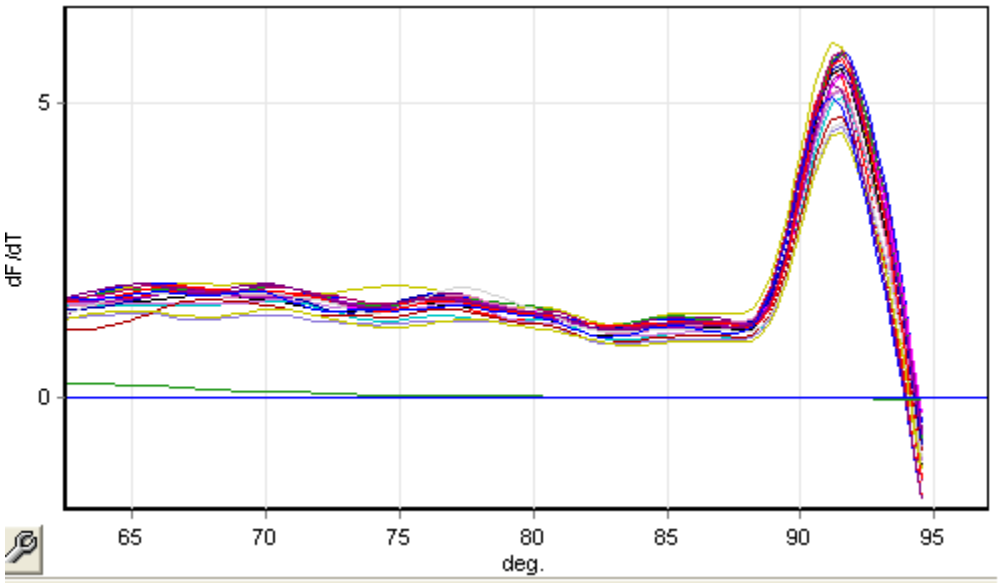
Figure_Apx 1: Standard curves of 10-fold serial dilutions displaying log concentration plotted on the X-axis and the cross point (Harris, Leese et al.) on the Y-axis. Rabbit globin cDNA standard curve .



Appendix B

Figure_Apx 2: Melt-curve analysis.

(A) Melt-curve analysis of rabbit globin cDNA.



(B) Melt-curve analysis of mtDNA